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Androgens, estrogens and progesterone concentrations in wastewater purification processes measured with capillary electrophoresis

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Abstract

A novel analytical-scale concept to improve reliability of detection and analysis of natural and processed wastewater samples from a purification plant was developed. A sequential sample clean-up system of polymer-based octadecyl and silane-based amino sorbents were used for concentrating human based steroid hormones and their metabolites and detecting them by UV absorption with capillary electrophoresis (CE). The 5-L water samples were collected from influent and effluent processes of the water purification plant in Helsinki, Finland.

The CE methods were partial filling-micellar electrokinetic chromatography and capillary zone electrophoresis. The analysis times and method concentration levels were optimised with eight steroids at the range of 0.5 mg/L – 10 mg/L. Since in CE the detectable quantities were higher than the existing amounts in the process waters, the real samples needed matrix removal combined with steroid enrichment. After 20000-fold concentration testosterone-glucuronide, androstenedione, progesterone, and estradiol-glucuronide could be determined in the process water samples. The amounts of individual steroids in influent and effluent waters were 11.3-425 ng/L and 0-151 ng/L, respectively. Correspondently, their total amounts were 745 ng/L and 285 ng/L with excellent in day and inter-day repeatability. The RSD values were less than 1%, 9.7%, and 19% in repeated analyses, in 60 analyses during 24 hours, and in 130 analyses during 15 months, respectively. The steroid removal in purification process was 65%. The solid particles separated in three steps during the water clean-up concept contained 9.8 ng/g - 45 ng/g steroids in combined dry precipitates.

Keywords: steroid hormones, influent water, effluent water, SPE enrichment, precipitate, capillary electrophoresis, UV detection.

1. Introduction

Environmental water contains liquid and solid residues, garbage, chemicals from living environment, excrements and residues from agriculture, institutions, hospitals, and commercial operators. They also may contain removal and emission waters of pulp and paper, mining, and biorefinary industries (Bennier 1999; Snyder 2008; Ort et al., 2010; Servos et al., 2007; Joblings et al. 2006; Whelton et al. 2015; Wilson 2013; Hashimoto 2000; Larsson et al. 2000)**[1-9]. P**harmaceutical and hormonal contaminants are at low concentrations in environmental water systems. According to many studies, especially steroid hormones were at ng/L level (Snyder 2008; Ort et al., 2010; Servos et al., 2007; Joblings et al. 2006; Metcalfe et al. 2003; Servos et al. 2005) [2-5, 10,11]. Steroids need continuous monitoring due to the harm they are noticed to cause in environment (Shargil et al. 2015; Scott 2013) [12]. The most studied steroids are estradiol (E2), progesterone, and testosterone, which in humans are found at 20-800 pg/mL, 0.02-4 ng/mL, and 0.3-250 ng/mL concentrations, respectively (Jones 1996; Vandenberg et al. 2012). [13, 14]. Based to the metabolism (Wikipedia 1, 2016) **[15]** VIITE Wikipedia 1, www.wikipedia.com/steroid, accessed 13.09.16] of steroids they are conjugated with glucuronic and sulphuric acids. In the body, they excrete to urine and further reach the environment. Especially, estrogens were noticed to exist at relatively high concentrations in warm water (Belhaj et al. 2015) [**19=16**]. It was also found that when E2 reached the environment, it caused feminization of animal species, physical abnormalities, and birth defects in fish (Vega-Morales et al. 2010) [**18=17**]. The reason for that are estrogen hormones, which are evidenced to disturb fecundity and hormonal activity of fish (Larsson et al. 2000; Tetreault et al. 2011) [9,**15=18].** Interesting is that male hormones and progesterone have not such a proven influence on hormonal activity of fish as the human female hormones have. As known from doping, the situation is different in humans. Therefore, estrogens hormones, especially estrone (E1) (Liu et al. 2009; Manickum et al. 2014; Vega-Morales et al. 2010;

Belhaj et al. 2015) [**16-19**] and estratriol (E3) need attention since E3 is metabolized from E1 and E2 in liver and placenta, and then excreted to environment. Therefore, wastewater pretreatment plants would need even more intensive processes to isolate estrogens (E1, E2 and E3) and their metabolites (17α-ethynylestradiol (EE2) (Manickum et al. 2014) [**17=20**], 17β-methoxy E2 and 17β−hydroxy E2) from the influent water than nowadays may be demanded (Giesbertz et al. 2016) [**21**] www.metabolia reittikaavio]. However, among estrogens the pregnancy hormone progesterone is detected in many wastewater systems but also in purified water effluents (Diniz et al. 2010; Nguyen et al. 2014; Carballa et al. 2004; Zarzycki et al. 2009) [**30-33**]. Progesterone has noticed to cause dose-dependent decreases in fecundity and fertility and significantly reduced gonadosomatic index and vitellogenin gene expression in females (Wojnarowicz et al. 2014; DeQuattro 2012). Its effect in fish is not exactly known.

The largest sources of released steroids contaminants for environmental water systems are thought to be wastewater treatment plants themselves and agriculture (Zhou 2012; Martz 2012) [**20=23, 21=24**]. Generally, the removal of contaminants is carried out with various kinds of processes in the plant. Methods like enzymatic hydrolysis with membrane bioreactors, mechanical treatment, aeration, nanofiltration, reverse osmosis, ozonisation, and filtration with activated carbon are used (Maletz et al. 2013; Al-Salhi et al. 2012) [**34=25, 35=26**]. However, water cleaning systems still need efficient evaluation since the steroid residues are found even in sludges (Azzouz et al. 2012 **OK**). In the wastewater treatment plants, the steroidal compounds are not totally removed from effluent waters. In addition, in the plant process the side-product is sludge, which unfortunately is not completely pure for recycling purposes (DeQuattro et al. 2012 **OK**).

[**22=28**]. Anyhow, the use of biological cleaning in the wastewater purification plants has grown. Yet, instead of decreasing the steroid concentrations, the amounts of some specific steroids were increased (Oller et al. 2011) [**36=29**]. In addition, steroids were formed during the water cleaning processes, since de-conjugation and reactivation of steroids was noticed to be catalysed by enzymes

(Mohagheghian et al. 2014; Spengler et al. 2001; Johnson et al. 2003; Shore et al. 2003; Buchberger 2011) [**23-28 =30-34**]. The use of enzymes produces cleaner water than without the biological handling. However, the fundament water chemistry may perhaps not be more closely applied although the idea is adapted. Presumably, the chemical reactions of microbe processes are solved, but plenty of commensurately research data are still needed to characterize the water systems and to obtain reference and methodologies in the steroid hormone analyses (Schröder et al. 2010) [**29=35**]. On the other hand, when steroids are detected in water, they may also be stabilized on solid material by complex formation with metal ions and other counter ions from soil. Following this the hormones may be deactivated (Zhang et al. 2013) [**37=36**], but when used as remedy of soil they may still be released to environment and migrate back into water systems. Therefore, to have the possibility for pre-empting the accumulation, it would be important to have accurate data about the existence of the steroid conjugates in water and in sludge. Usually, steroids in water samples are studied by gas chromatography (GC) and liquid chromatography (LC) (Petrović et al. 2005) [**38=37**]. However, capillary electrophoresis (CE) has also utilized because of the compound targeted possibility to use chemical and instrumental modifications (Nyakubaya et al. 2015; Görög 2004) [**39=38,40=39**]. As to environmental waters, it lacks sensitivity. It is well known that CE gives better efficiency than LC in separation of structurally similar compounds (Nyakubaya et al. 2015) [39]. GC-MS method has shown to be sensitive to estrones in wastewater samples where estradiol (100 mg/L) and estriol (54 ng/L) were found in one influent sample, only (Andrási et al. 2011) [**39=38**]. Earlier however, in the wastewater treatment plants their concentrations were from less than 10 ng/L to nearly 1200 ng/L (Görög 2004) [**40=39**]. In that case, CE cannot be used without concentration enhancement, although the possibility is to use sample stacking prior to separation (Sirén et al. 2015) [**41=40**]. Generally, in CE concentration increase can be achieved by 370-fold to improve the detection response, which however is not enough in wastewater analyses when an UV detector is used (Monton et al. 2014; Sihvonen et al. 2014; Amundsen et al. 2004; Sirén et al. 2008 & 2014;

Aufartová et al. 2011) **42=41 43=42-47=46**]. Especially, partial-filling micellar electrokinetic capillary chromatography (PF-MEKC) with ionic surfactant and micelle solutions have shown to allow concentration behaviour for non-ionic compounds (Carabias-Martínez et al. 2000). Then, the method utilizes surfactants, which are used to form pseudostationary micelle plugs in the capillary. Especially, when the plug is positioned in the inlet end of the capillary behind the electrolyte solution, the viscosity difference is advantageous in respect of non-ionic steroid concentration. Because the micelles have roughly a spherical structure and a hydrophobic interior with a hydrophilic exterior combination, the analytes interact with the micelles and separate according to their partition in the electrolyte solutions.

The purpose of the present work was to study human based steroids in intake water of the water purification plant. In addition, the work was done to determine the steroid concentrations and to compare the efficiency of the SPE pre-treatment. The final aim was to develop an analytical scale sample clean up and centration method for detecting steroids in wastewater purification process waters with CE and UV. As a result, the data was assumed to give general remarks and demonstrate the need of intensive purification and water control system in wastewater purification plants. Therefore, an intensive isolation of steroids from the water and sludge of Helsinki wastewater purification plant are demonstrated with partial-filling micellar electrokinetic chromatography (PF-MEKC) and UV detection. The profound methodology to clean the samples and isolate the steroid with solid-phase extraction (SPE) methodologies was developed using both nonpolar and amine sorbents. The targeted study was focused to detect the endogenous androgens, estrogens and progesterone in the water samples from the wastewater purification plant in Helsinki where the influent water is received from Baltic Sea (Gulf of Finland). The plant serves the purified water to 800 000 people. According to our literature search, the overall analytical concentration process with the CE methods is not introduced earlier.

2. Experimental

2.1. Chemicals

Fluoxymesterone (4-androsten-9α-fluoro-17α-methyl-11β, 17β-diol-3-one, $C_{20}H_{29}FO_3$, MW 336.44 g/mol, TLC grade 1), 4-androsten-17β-ol-3-one glucosiduronate (testosterone-glu, *T-gluc*, $C_{25}H_{36}O_8$, MW 464.55 g/mol, TLC grade 1), 1,3,5(10)-estratrien-3,17-β-diol 3-glucosiduronate (estradiol-gluc, *E2-gluc*, $C_{24}H_{32}O_8$, MW 448.51 g/mol, TLC grade 1), 3-hydroxyestra-1,3,5(10)-trien-17-one glucuronide (estrone-gluc, *E1-gluc*, $C_{24}H_{30}O_7$ MW 446.50 g/mol, TLC garde 1), and 1,3,5(10)-estratrien-3,16α,17β-triol glucuronide (estriol-gluc, *E3-gluc*, $C_{24}H_{32}O_8$ MW 464.52 g/mol, TLC grade 1) were purchased from Steraloids (Newport, RI, USA). 17α-methyltestosterone ($C_{20}H_{30}O_2$, MW 302.45 g/mol, HPLC ≥ 98%) was from Riedel-de Haën (Seelze, Germany). Androsterone ($C_{19}H_{30}O_2$, MW 290.44 g/mol, HPLC ≥ 97.6%), 17α-hydroxyprogesterone ($C_{21}H_{30}O_3$ MW 330.46 g/mol, assay ≥ 95%), progesterone (*Prog*, $C_{21}H_{30}O_2$, MW 314.46 g/mol, assay ≥ 98%), testosterone ($C_{19}H_{28}O_2$, MW 288.42 g/mol, assay ≥ 98%), and androstenedione (*Andr*, $C_{19}H_{26}O_2$, MW 286.41 g/mol, assay ≥ 98%) were from Sigma-Aldrich (Germany).The steroids were used as received. They were stored in a dark and cold room (+4 ^{o}C).

Other chemicals were ammonia (min. purity 25%) from VWR International S.A.S (France), 3-[cyclohexylamino]-1-propane-sulfonic acid (purity ≥ 98.0%, CAPS), and ammonium acetate (98%, AA) from Sigma-Aldrich (Germany), diethyl ether (GC assay, min 99.5%) from Merck (Germany), orto-phosphoric acid (85%, acidimetric assay, 85.0-88.0%) from Merck (Germany), buffer solutions of pH 4, pH 7, and pH 10 (made of phthalate, phosphate, and borate, resp.) and methanol (HPLC grade) from Fisher Scientific (UK), and ethyl acetate (GC assay > 99.5%) from Sigma-Aldrich (Germany). The sodium salt of taurocholic acid monohydrate (BioXtra, ≥ 95% (TLC)) and sodium dodecyl sulphate (approx. 99%, SDS) were from Sigma-Aldrich (Germany). Hydrochloric acid (1.0 M, analysis result 0.9995 mol/L, ±0.0021 mol/L) and sodium hydroxide (1M, analysis result 1.0003 mol/L, ± 0.0021 mol/L) were from Oy FF-Chemicals Ab (Finland). Methanol was used as the solvent in preparation of standards and as the marker of electroosmosis in CE.

2.2. Instruments

Capillary electrophoresis separations were made with a Hewlett-Packard 3^D CE instrument (Agilent, Waldbronn, Germany) equipped with a photodiode array detector (λ 190-600 nm). The CE instrument was applied with ChemStation programmes (Agilent) for instrument running and data handling. Bare fused silica capillaries (i.d. 50 µm, o.d. 375 µm) were purchased from Polymicro Technologies (Phoenix, AZ, USA). They were cut to the total length (L_{tot}) of 80 cm and the effective length (L_{eff}) of 71.5 cm. Before use they were conditioned by sequentially flushing with 0.1 M NaOH, milli-Q water, and the electrolyte solution, for 20 min each at high pressure 2.0 p.s.i. (140 mbar).

The temperature during the PF-MEKC and CZE analyses was +25 °C. In all cases, positive polarity and voltage of 25 kV was set as the constant value. The electrolyte solutions in both techniques gave a stable 17 µA current which was monitored during all analyses. Simultaneous peak detection was made at 214, 220, 240, 247, and 260 nm in PF-MEKC and at 200, 214, 240, 247, and 254 nm in CZE methods. In PF-MEKC the steroids were detected at 247 nm except estrogens at 214 nm. On the contrary, in CZE the glucuronide conjugates were detected at 200 nm (E1-gluc, E2-gluc, and E3-gluc) and at 247 nm (T-gluc).

In the PF-MEKC-UV method the micellar solution was introduced at 0.50 p.s.i (34.5 mbar) for 75 s (volume in hydrodynamic injection was 55.67 nL, CE Expert Lite, SCIEX). After the micellar plug, the sample was introduced at 0.725 p.s.i. (50 mbar) for six seconds (volume in hydrodynamic injection was 6.46 nL) from inlet of the capillary towards the detector. *In the CZE-UV method* (electrolytes CAPS and AA), the sample was injected with 0.725 p.s.i. (50 mbar) for 6 seconds.

Before each analysis, the capillary was flushed with 0.1 M NaOH and the electrolyte solution for 2 min and 5 min, respectively. After every eight runs (only for very dirty water samples and long sequences), the capillary was washed with 0.1 M NaOH, milli-Q water, and the electrolyte solution for 7 min, 5 min, and 10 min, respectively.

2.3. Other devices

The pH of the electrolyte solutions were adjusted using an InoLab pH7110 (WTW) instrument, which was calibrated with buffers of pH 4.00, 7.00 and 10.00 (Fisher Scientific, Loughborough, UK). The samples were centrifuged with a MSE MISTRAL 1000 instrument at 2000 rpm and mixed with a Vortex-Genie 2 device (Scientific Industries Si, Prolab-Oriola Oy, Finland). The buffer solutions were made with a Branson 5510 ultra-device. Chemicals were weighted with a Sartorius AG balance (BP 301 S). The SPE device Vac Master was used for solid-phase extraction and concentration of the samples. The SPE filtrates were evaporated under N_2 gas with an evaporation unit (Thermo Scientific, Vantaa Finland). All water used were purified with a Direct-Q UV Millipore water purification system (Millipore S.A., Molsheim, France).

2.4 Filters and solid-phase extraction sorbents in sample preparation

The water samples were filtrated with glass microfibers and membrane filters. They were purchased from GE Healthcare Life Sciences (Whatman™, Glass Microfiber Filters GF/C™, Diameter 90 mm) and Millipore (Durapore® Membrane Filters, 0.45 μm HV), respectively. Next, the water samples were concentrated with Strata-X 33u polymeric reversed phase columns (reverse phase, 500 mg / 6 mL, U.S.A.) for non-ionic compound extraction and with amino (NH_2) polar phase columns (3 mL, quaternary amine (N^+), amine silane 40 μm APD, 60 Å) purchased from J.T. Baker Inc. (The Netherlands) for extraction of glucuronide conjugates. The Strata-X sorbent relies on three mechanisms of retention: π–π bonding, hydrogen bonding (dipole-dipole interactions), and hydrophobic interaction. The amino sorbent is based on ion exchange mechanism of the anionic functional group between the analytes.

2.5 Preparation of electrolyte solutions

2.5.1 Electrolyte in PF-MEKC-UV separations

The electrolyte solution in the partially filled micelle zone composed of 20 mM ammonium acetate. Its pH was adjusted with 25% ammonia to pH 9.68. It was developed for determination of corticosteroids and metabolites in patient urine samples (Sirén et al. 2008). Preparation of the stock

206 solutions of sodium dodecyl sulphate (SDS) and sodium taurocholate were made into the ammonium
207 acetate and milli-Q water, respectively. They both were stored at room temperature in separate glass
208 vessels. Neither electrolyte nor the micelle solutions were filtered before use, but instead, they were
209 degassed in an ultrasonic bath for 15 min at room temperature.
210 The final micelle solution was prepared by mixing 1000 μL of 20 mM ammonium acetate (AA)
211 solution, 440 μL of 100 mM SDS in 20 mM AA, and 50 μL of 100 mM sodium taurocholate solution
212 together, in this specific order. The micelle and the electrolyte solutions were sequentially introduced
213 into the capillary, but first the electrolyte and followed by the micelle. Then, the micelle plug was
214 placed between the electrolyte solution and the standard or the sample solution.

215 **2.5.2 Electrolytes in CZE-UV separations**

216 The CAPS electrolyte solution was prepared from 15 mL of freshly made 0.2 M CAPS in milli-Q
217 water, 20 mL of 0.1 M NaOH in milli-Q water, and 15 mL of milli-Q. The volume percentages were
218 30, 40, and 30, respectively. It was modified from the CAPS electrolyte published earlier (Riekkola
219 et al. 1996). [Riekkola ML and Jumppanen JH, Capillary electrophoresis of diuretics, Journal of
220 Chromatography A 735 (1996) 151-164.] The ammonium acetate (AA) electrolyte was prepared as
221 described in Ch. 2.5.1.

222 **2.6 Sampling collection and pretreatment**

223 Water samples from the wastewater purification plant in Helsinki were collected at three occasions
224 (sampling on 2014, March; 2015 April; 2015 August). The accredited personnel of the wastewater
225 treatment plant made the sampling from the influent and effluent waters into 5 L-volume plastic (high-
226 density polyethylene) containers. The samples were from the influent water, and from the effluent
227 water after the bio filter unit (combined aerobic and anaerobic process). No modifications were done
228 after the sampling because the water samples were immediately delivered to laboratory. They were
229 filtered, separated into portions, extracted, and concentrated for capillary electrophoresis analyses.
230 For one sample, the non-stop process with the analytical scale sorbent columns took three days per

one sample of five litres. Then the 250 µL concentrate was ready for capillary electrophoresis analysis.

2.7 Extraction and preconcentration of analytes

2.7.1 Extraction from aqueous fraction

In the laboratory the influent and effluent water samples were homogenized, filtered, and divided in 1 L and 2 L sample portions, which were the main samples. The influent water was analysed separately from the effluent water. They both solvents were filtered through fiberglass filters (pore size of 0.45 µm) and then through 0.45 µm membrane filters. Next, the filtrates were pre-treated with SPE and the isolated steroids were on Strata-X material. Before use, the SPE columns were washed with methanol and water. Then the samples were introduced onto the sorbent by pumping the sample solution at the flow rate of 8 mL/min. After that, the SPE materials were dried in vacuum for 30 min. Extraction of the steroids from the non-ionic polymer material was made with methanol or ethyl acetate (3 x 1 mL). The studies were performed with 3 replicates and with three sequential analyses. The whole procedure is shown in **Figure 1**.

The SPE filtrate from the Strata-X was further concentrated with amine sorbent for studying the quantitatively of the Strata X extraction and in order to monitor the efficiency of steroid glucuronide retaining. After the sample sorption and release, the steroids were extracted into diethyl ether. After elution the solutions were evaporated under nitrogen at 40 ^{o}C temperature and the precipitate was dissolved into 250 µL of methanol for the CE analyses.

2.7.2 Extraction from filtrates and solid fraction

The filtrates and the solid residues collected on the filter membranes (processed with post and pre SPE steps) were studied separately. The precipitates left from the water samples of Helsinki wastewater purification plant on the filtration paper, were studied with elemental analysis (Graphite 120s). According to the measurements the dry solids contained 2.7-6.1% nitrogen (N factor 0.988) 39-41% carbon (C factor 0.994), and 5.8-6.1% hydrogen (H factor 0.990). It did not contain sulphur

256 (S factor 1.043). The precipitate was also dissolved into methanol, because of the capillary
257 electrophoresis analyses (**Figure 1**). Then, as explained above the solutions were evaporated under
258 nitrogen and after methanol evaporation the solids were dissolved back into methanol (250 μL)
259 before analyses.

260 **2.8 Optimization of the separation parameters**

261 In this study, the PF-MEKC-UV method for corticosteroids (Sirén et al. 2008) was optimized for
262 androgen, estrogen, and progesterone hormone separations. Testosterone and androsterone were used
263 as the model compounds, since estrogen compounds had very low sensitivity at 247 nm in PF-
264 MEEKC. In addition, because progesterone appeared always a very high and identifiable peak in the
265 electropherograms, androsterone was selected on behalf of it. The chemical and instrumental
266 optimization of the CE methods were made with injection pressure and time, concentration and pH
267 of the electrolyte solution, capillary dimensions, applied electric field, temperature, and
268 concentrations of both SDS and sodium taurocholate in the micelle solution with a specific pH. In
269 addition, the impact of different preconditioning methods before analyses were tested.
270 The CZE methods were also optimized as the PF-MEKC method (Amundsen et al. 2008; Sirén et al.
271 2008). The CAPS and AA electrolytes were chosen by paying especially attention to the separation
272 efficiency and the sensitivity of the steroid glucuronides.

273 **2.9 Preparation of standard solutions**

274 The stock solutions of steroid hormones at 1000 μg/mL were prepared in methanol and stored at +4
275 ˚C. The working solutions were prepared from the stock solutions and diluted with methanol. When
276 stored in cold, the solutions were let to warm up to room temperature before use. Furthermore, before
277 dosing the solutions they were mixed with a vortex mixer.

278 **2.9.1 Standards in concentration calibrations with <u>external standards</u>**

Calibration for SPE fluids (PF-MEKC): The steroid concentrations were 0.5 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 6 μg/mL, 8 μg/mL, and 10 μg/mL for T-gluc, androstenedione (Andr), and progesterone (Prog). Otherwise the measurements were made at 0.5-10.0 μg/mL level.

Studies of glucuronides with SPE treatment (CZE - CAPS and CZE - AA electrolytes): The concentrations of 0.1 μg/mL, 0.2 μg/mL, 0.3 μg/mL, 0.4 μg/mL, 0.5 μg/mL, and 0.6 μg/mL were used for determination of T-gluc and E2-gluc in the water samples.

2.9.2 Standards in the ***method of standard addition***

Calibration for SPE fluids (PF-MEKC): T-gluc, androstenedione (Andr), and progesterone (Prog) from influent filtrate were quantified by standard addition method. The sample volume SPE filtration was always 100 μL in each case. Methanol was used to fill up the solutions to the desired total volume.

Calibration for SPE fluids (CZE - CAPS and CZE - AA electrolytes): After standard addition to the influent filtrates the concentrations of T-gluc and E2-gluc were 0 μg/mL, 2 μg/mL, 4 μg/mL, 6 μg/mL, and 8 μg/mL.

3 Results

Pitää vielä laittaa että MeOH on parempi uuttoliuotin kuin EA. Ja että CAPS on parempi CZE puskuri kuin AA, koska se toimii konsentroivasti steroidien erotuksessa.

3.1 Method optimization

The analyses with capillary electrophoresis were divided into two parts based on the ionization of the steroids. The non-ionic androgen steroid hormones (pKa 19.04-19.09) and progesterone (pKa 18.92) were determined with partial-filling micellar electrokinetic chromatography (PF-MEKC). The anionic steroid glucuronide conjugates (pKa 3.30, glucuronic acid, (Wikipedia, accessed 15.10.16) http://www.chemicaldictionary.org/) were determined with capillary zone electrophoresis (CZE) using 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS) and ammonium acetate (AA) electrolytes. First, non-ionic steroid compounds and steroid glucuronides were analysed individually and their migration order was measured. Then, the methods were used for separation of the steroids

306 in mixtures in order to optimize the overall methodology based on compound resolution and detection
307 limits. Since UV detection was used, identification of the steroids was done by migration times. In
308 addition, their recognition was done compound by compound by fortifying the samples with a two
309 μg/mL standard. Each individual steroid was further spiked to the SPE-extracts for monitoring the
310 efficiency of the purification and for detecting the compounds left in the remains. In the applications,
311 androgens, estrogens and progesterone were studied both from process waters and from filtrates. They
312 were also studied from solid material precipitates in the water handling made in laboratory **(Figure**
313 **1).**
314 In PF-MEKC, a discontinuous electrolyte solution combination was used. It was made of taurocholate
315 and sodium dodecyl sulphate and of ammonium acetate (AA) solutions (Sirén et al. 2008).
316 Androsterone that was not detected in real samples and testosterone were used as the model
317 compounds to optimize the method, since their sensitivities and electrophoretic mobilities were
318 affected by changes of chemical and instrumental parameters. Under the optimized conditions, the
319 steroids migrated in the order of testosterone glucuronide (T-gluc), fluoxymesterone,
320 androstenedione, testosterone, 17α-hydroxyprogesterone, 17α-methyl testosterone, and progesterone
321 (***Figure 2A***).
322 With CZE methods, four conjugates E1-gluc, E2-gluc, E3-gluc, and T-gluc were detected and
323 separated. The androgen metabolite T-gluc was selectively detected at 247 nm whereas estrogens
324 absorbed UV light only at the 200 nm wavelength. In CZE - AA method E3-gluc did not give any
325 peak (***Figure 2B***) being at 0.5-20 μg/mL concentrations. Therefore, CZE-CAPS method was used for
326 its determination (***Figure 2C***). The two CZE methods were used in quantification of the steroid
327 metabolites extracted from the real water samples and the solid particle matrices. Overall, CAPS
328 buffer was more efficient solution than the basic AA buffer in the electro aided separation, because
329 it could also be used for separation of the estrogens conjugates with good UV sensitivity (***Figure 2B***).

In addition, the quantitative results obtained with PF-MEKC and CAPS correlated. The drawback of CAPS electrolyte was its interaction with sample matrix that reduced the sensitivity.

The PF-MEKC was the most repeatable of the three methods regarding to the separation of the endogenous steroid hormones and testosterone glucuronide. The correctness of the results was verified by the relative standard deviations of the absolute migration times of individual steroids, their electrophoretic mobilities, and the mobility of electro osmosis, which were 1.4-4.2 % (RSD 1.4-3.6 % in a mixture), 0.6-5.0 % (RSD 1.6-10 % in a mixture), and 1.5 %-3.8 % (RSD 1.4-3.8 % in a mixture), respectively (***Table 1A***). The reproducibility of the PF-MEKC method was between 0.6% (androstendionone) and 5.1% (T-gluc). Without the micelle, in CZE - CAPS the RSD of absolute migration times were 2.3% (E2-gluc) and 6.4% (T-gluc). Furthermore, in the CZE - AA solution the migration times, the electrophoretic mobility values, and the mobility of electroosmosis were 10 %, 9.5 %, and 5.9 %, respectively (***Table 1B***).

The PF-MEKC results also showed that the inter-day precision calculated from the absolute migration times was reproducible. The accuracy (RSD %) was 1.5% and 2.5% in the two AA based electrolytes and 1.1 in CAPS. The average values of inter-day precision values were 4.9, 10, and 6.7 % (RSD %) in PF-MEKC, CZE-AA, and CZE-CAPS, respectively.

The steroid standard mixtures were used with in-day analyses, which were measured for five times with five repeated analyses per day (***Table 1 A and B***). The inter-day reproducibility was measured for 105 times with the standards and for five times in each sequence during 7 days in 3 months (5 x 7 x 3 times) before the real water samples were determined. The results were calculated from the average of absolute migration times of the electroosmosis marker methanol (first compound) and progesterone (last compound). In PF-MEKC separation, the precision values (RSD %) were less than 1 %, 10 %, and 19 % in repeated analyses, in 60 analyses made within 24 hours, and in 130 analyses during 15 months, respectively. The ruggedness and the robustness were obtained under a variety of conditions by changing the capillaries, temperature, renewal of the preparation of electrolytes and

355 micelle solutions, pH, ionic strength, methanol concentration, batches of the SPE sorbent, and the
356 water sample matrices.

357 **3.2 Linearity and sensitivity**

358 The concentration ranges for quantification of the steroids were calibrated with their mixtures. In
359 general, the correlation coefficients for all compounds were better than 0.95 (r^2) (***Table 2A***). In PF-
360 MEKC, the LOD (S/N 3) and LOQ (S/N 10) values of the steroid hormone standards were from 0.03
361 to 0.5 μg/mL and from 0.08 to 1.50 μg/mL, respectively. This meant that the concentrations of the
362 steroids in the water samples needed enhancement to fulfill the method-related quantification range.
363 Therefore, SPE system was considered to achieve the minimum quantity of 1.5 ng/L for T-gluc in
364 PF-MEKC and successful determination of T-gluc and E2-gluc in CZE with acceptable precision and
365 accuracy. Although the specificity was fulfilled and the method was able to measure accurately and
366 specifically the analytes of interest, the other estrogen glucuronides could not be identified in the
367 water extracts. As listed in ***Table 2B and C*** two glucuronides were determined in the CZE methods
368 using both CAPS and AA. Then the repeatability for T-gluc was 0.950-0.996 and 0.951-0.985 (R^2),
369 respectively.

370 **3.3 Efficiency of solid phase extraction to concentrate the steroids**

371 Purification processes of the effluent waters of the wastewater purification plants differ from each
372 other (Sirén et al. 2016) [VIITE: Sirén, Heli; El Fellah, Samira, Steroids contents in waters of
373 wastewater purification plants: determination with partial-filling micellar electrokinetic capillary
374 chromatography and UV detection, International Journal of Environmental Analytical
375 Chemistry (2016), 96(11), 1003-1021.]. Therefore, efficient and accurate sample preparation
376 methods needed to cover selectively a wide range of steroid hormones and their conjugated species.
377 Moreover, selective sample clean-up is needed since the steroids existed at very low concentrations
378 (Aufartová et al. 2011) [48]. In the present study, the steroids from the analytical samples were at the
379 volume of 1 L that is accordance with 0.5 – 1 L samples described in the literature (Trinh et al. 2012;
380 Mompelat et al. 2009) [49, 50]. The difference was that the samples from the plant water taken from
381 purification processes were larger (5 L) which were divided to analytical samples that could be
382 compared with each other. The pretreatment procedure described in the study is a new proposal for
383 the laboratory-scale cleaning and enrichment of the steroids and their human based metabolites from
384 real water samples when the detection is made with UV **(Figure 1).** The procedure was modified
385 from the SPE methodology used in doping control of steroids in human urine samples (Kolmonen et
386 al. 2007) [51]. Usually, the glucuronide conjugates are hydrolysed to free the parent compound for
387 analysis. In our study, the hydrophilic steroid glucuronides needed only intensified trapping and
388 isolation from the waters. They were not manipulated with enzymatic treatment, why they were, as
389 they existed in the plant water.
390 The sufficiency of the water pre-treatment was thoroughly investigated with the influent and effluent
391 waters of the Helsinki wastewater purification plant. The Strata-X and amino sorbents (Table 3)
392 showed very good capacity to retain the steroids of the study. The results show that SPE treatment
393 had a role in the quantitative results, since the steroid elution from Strata-X differed significantly
394 between extractions made with methanol and ethyl acetate. From those two, methanol eluted more

steroids from the sorbents and the studied compounds could be identified without the standard addition for authentication.

However, according to the results of the present study the retention with non-covalent (electrostatic), hydrogen bond (dipole-dipole), and hydrophobic interactions on Strata-X were not as good as considered for quantitative extraction of human steroids. The filtrate eluted during the steroid sorption procedure contained anionic steroids as shown by handling the eluted water phase with quaternary amine sorbent, the performance of which is based on the ion exchange interactions (Figure 1). The method validation showed that the SPE treatments with Strata-X and amino sorbents were sufficient enough to make the concentrates from the real water samples (Table 2B). The new extraction methodology was extremely good for isolation of matrix background. Overall, the solid phase materials concentrated the steroids by 20000-fold enabling their determination with capillary electrophoresis combined with UV detection. The precipitates and solid particles in the water samples were isolated during the steroid concentration steps and studied similarly as the water phases (Figure 1).

3.4 Water samples

Almost 80% of the samples from effluent wastewater treatment plants contain female hormones (Johnson et al. 2003; Shore et al. 2003) [26,27]. In many cases, these steroids cannot be detected efficiently due to the small sample volumes used as the analytical samples. It is known that healthy male persons form testosterone 6–10 mg/day. Testosterone is glucuronate and sulphate conjugates at 40 ng/mL and 5 ng/mL concentrations (Sten et al. 2009) .[REF Taina Sten, Ingo Bichlmaier, Tiia Kuuranne, Antti Leinonen, Jari Yli-Kauhaluoma, and Moshe Finel, UDP-Glucuronosyltransferases (UGTs) 2B7 and UGT2B17 Display Converse Specificity in Testosterone and Epitestosterone Glucuronidation, whereas UGT2A1 Conjugates Both Androgens Similarly, Drug Metabolism and Disposition 37 (2009) 417–423). The results of the study show that the influent and effluent water samples contained notable amounts of testosterone glucuronide, androstenedione, and progesterone

(Figure 2, Table 3). Identification was done by migration time compatibility and identifying with the standard addition for peak authentication. Previously, estrogenic hormones were earlier noticed at significant concentrations only in influent waters (Görög 2004) [40 =??]. However, in the present study, the quantities of E2-gluc were high in both influent and effluent waters varying from 11.3 ng/L to 74.8 ng/L depending on the eluent in SPE and the CZE method used in determination. The concentration of E2-gluc was noticed to increase during the pretreatment process in the plant. This is accordance with the literature since steroid hormones are known to have structural changes by enzymes used in biological treatment of wastewater treatment plant (Kolmonen et al. 2009). Estrogenic hormones can be detected at low concentrations as 6 ng/L in surface waters which is reported. Women excrete them at 2-12 µg/person/day (Belfroid et al. 1999) [A.C.Belfroid, A. Van der Horst, A.D.Vethaak, A.J. Schafer, G.B.J. Rijs, J. Wegener, W.P. Cofino, Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands, The Science of the Total Environment 225 (1999) 101-108.] In environmental water systems the estrogens concentration is not allowed to exceed 1 ng/L, which is the level that may cause estrogenic effects in aquatic organisms (Esteban et al. 2014) [VIITE: Sci Total Environ. 2014 466-467:939-51. Analysis and occurrence of endocrine-disrupting compounds and estrogenic activity in the surface waters of Central Spain. Esteban S[1], Gorga M, Petrovic M, González-Alonso S, Barceló D, Valcárcel Y.] Several estrogens were also detected in the sources of drinking water treatment plants but not in the finished water (Benotti et al. 2009) (Mark J. Benotti, Rebecca A. Trenholm, Brett J. Vanderford, Janie C. Holady, Benjamin D. Stanford and Shane A. Snyder, Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water, *Environ. Sci. Technol.*, 2009, 43 (3), 597–603. The experiments show that the steroids in wastewater need to be studied very comprehensively. In our study, their values were very small and the PT-MEKC method could not be used. by women E2-gluc was only quantified with CZE-CAPS and CZE-AA methods. Its amount was 11.3 ng/L and 22.7 ng/L in the influent water samples **(Figures 3).** The other

445 detected steroids, testosterone-glucuronide, androstenedione, and progesterone were at 77.5-120
446 ng/L, 247.7-284 ng/L and 0.0-128.3 ng/L concentrations in the influent water and at 8.3-43.5 ng/L,
447 53.5-171 ng/L, and 0-4.8 ng/L, respectively, in effluent water (**Table 3**).The calculations are in
448 correlation with the results published earlier in literature (Nyakubaya et al. 2015)[39=???]. The
449 novelty value of the new method presented here is that the concentrations of testosterone-
450 glucuronide and androstenedione, which are the metabolites of both testosterone, and progesterone,
451 could be measured at the same time (**Figure 2**). However, the other androgenic steroids were not
452 observed. Our results also showed that the pretreatment procedures in the wastewater purification
453 plants remarkably decreased the amounts of the steroids, but especially that of progesterone. The
454 processes do not completely isolate the steroids from effluent, why they would need more efficient
455 controlling and extra purification before discharge into environment or used for other purposes.
456 **Table 3** shows that by PF-MEKC and CZE-CAPS methods, the total steroid quantity in the influent
457 water was 526 ng/L, which was calculated from the joint quantities of testosterone glucuronide,
458 androstenedione, progesterone, and E2-gluc purified with amine and Strata sorbents and eluted with
459 ethyl acetate. Naturally, their total steroid amount in the effluent water was much lower than in
460 influent being 126 ng/L. The reason for high concentration of androstenedione may be that
461 progesterone can produce androstenedione that is the precursor in metabolism of testosterone (one of
462 the androgens) and estrogens such as estradiol (Nyakubaya et al. 2015) [39]. Otherwise, the removal
463 of the individual steroids were much lower. In our study, the removal of E2-gluc did not occur
464 calculated from the results measured from influent and the effluent water samples, although the
465 deletion of T-gluc, androstenedione, and progesterone were 76%, 65%, and 100%, respectively.
466 **3.5 Precipitates from water filtrates**
467 E2-gluc, T-gluc, and androstenedione were the most dominate steroids also in the precipitates formed
468 in filtration of the influent and effluent waters when preparing them for analysis **(Figure 4, Table 4)**.
469 Based on the present data, higher concentrations were observed when the waters were filtrated

immediately in laboratory (pre-SPE precipitate). The amounts were also significant in the precipitates after the eluents were filtrated after SPE pre-treatment (post-SPE precipitate).

According to literature the biofilm purification (biomembrane or biorotor) used in the WWTP degreases estrogenic compounds on average only by 28% compared with the methods using active sludge purification, which has observed to reduce the quantity by 81% (Andersen et al. 2003) [52=???]. Filtrates and precipitates of the purification plant waters contained the solid particles as the precipitate 9.8 ng/g - 45 ng/g depending on the methods used in dilution, sample preparation and determination. E2-gluc was at 64% on the particles. On the contrary, T-gluc, androstenedione, and progesterone were at 40 %, 36 %, and 40% on the particles.

4 Conclusions

In this paper, micellar electrokinetic chromatographic and capillary zone electrophoresis methods were applied to the determination of human steroid hormones and metabolites in influent and effluent waters of the water purification plant producing drinking water in Helsinki area. In addition, they were used to measure the steroid amounts in the particles precipitated on the membranes during water filtration. Capillary electrophoresis techniques showed to work excellent for comprehensive profiling of the androgens, estrogens, and progesterone steroids after solid phase extraction enrichment. Due to low amounts in in influent and effluent and the low UV sensitivity, the steroids needed to be extracted from 1-L water volume. With respect to the simple separation conditions and miniaturization benefits of the proposed CE techniques, it may be prominent and reliable alternative to conventional laboratory GC-MS and LC-MS for analysis of steroids.

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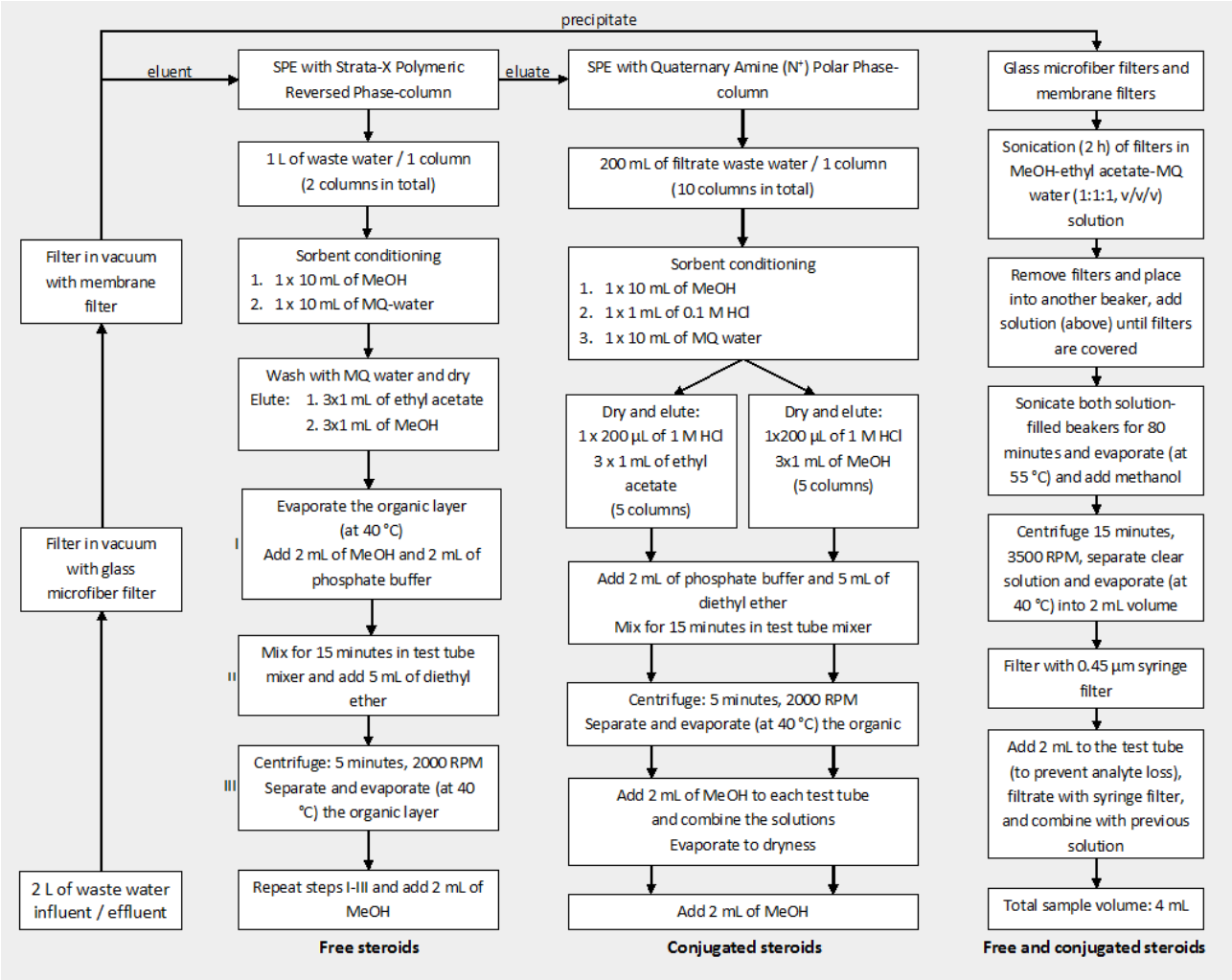

Figure 1. Scheme of sample preparation of influent and effluent water samples and soluble particles. Separation of free steroids and conjugated steroids with solid-phase extraction and solid-liquid. extraction

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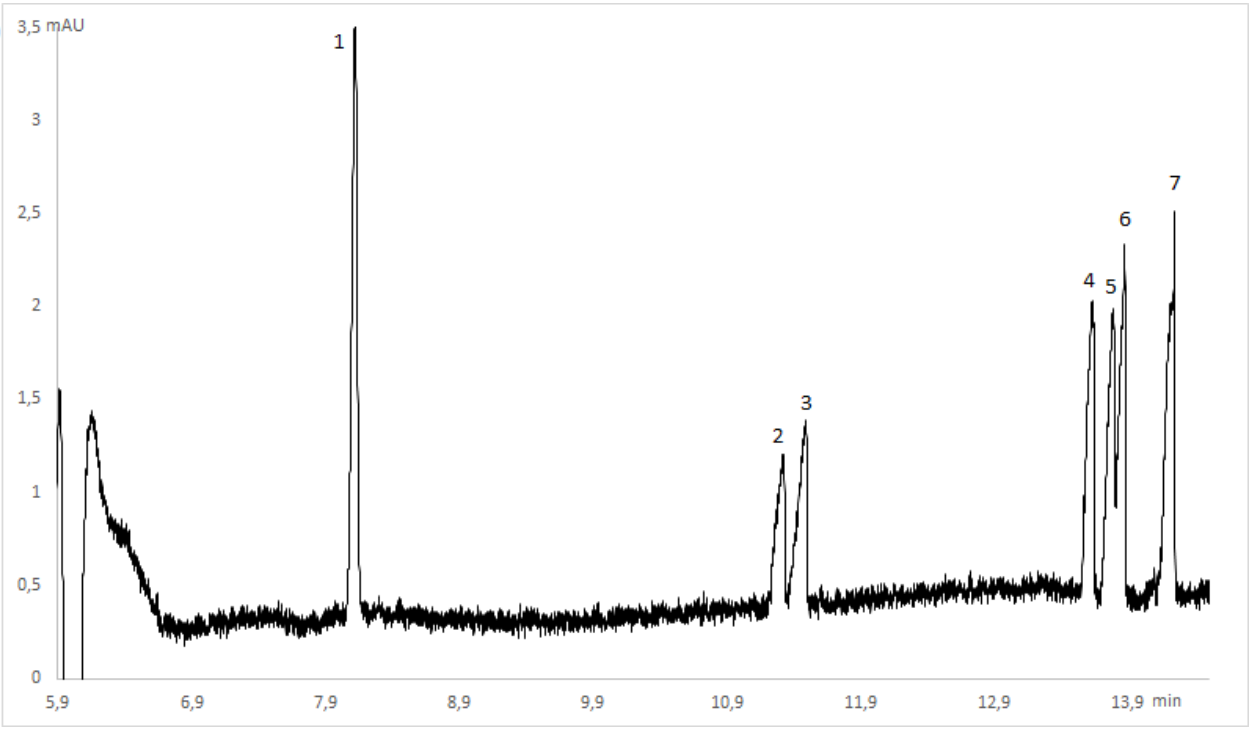

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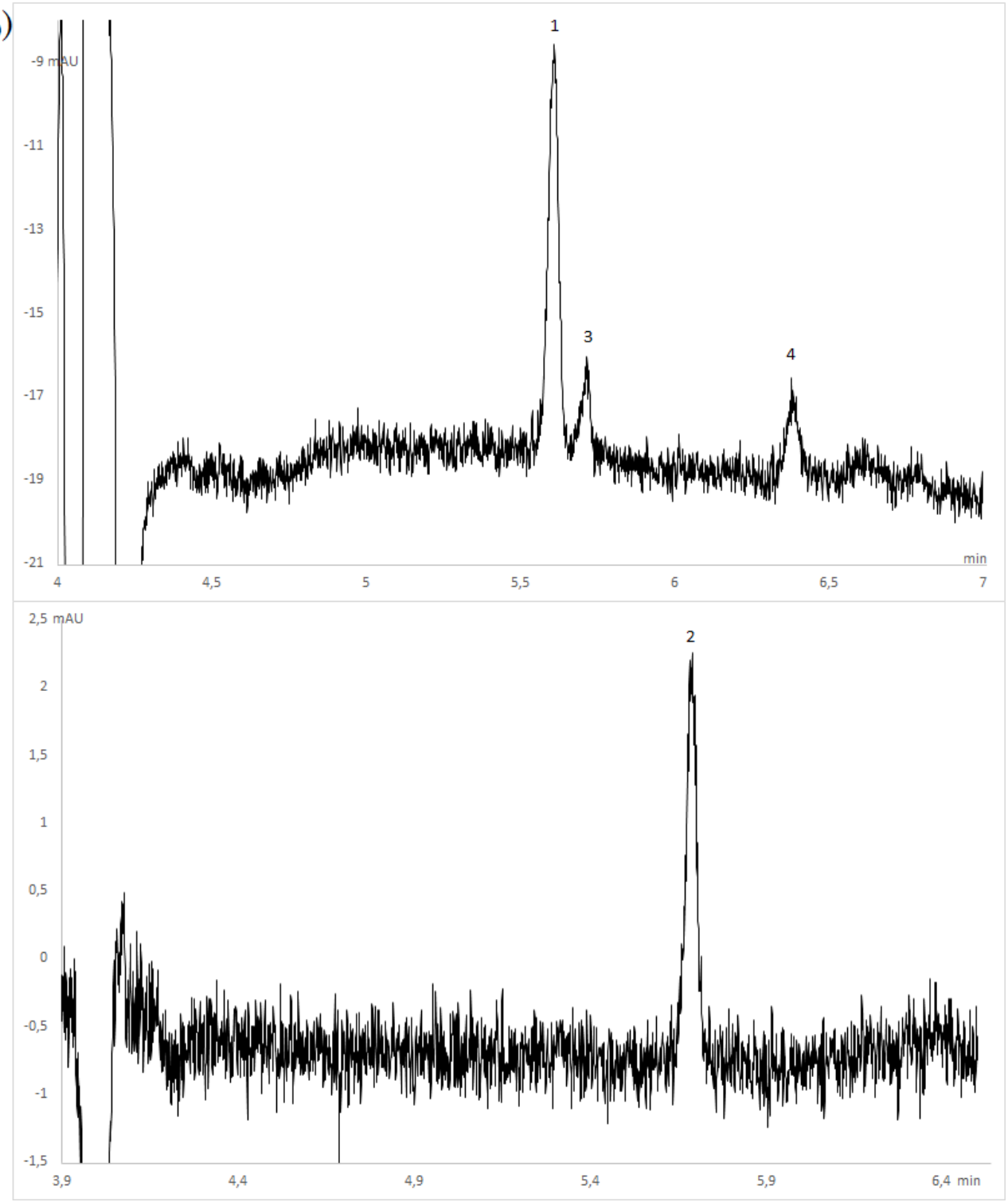
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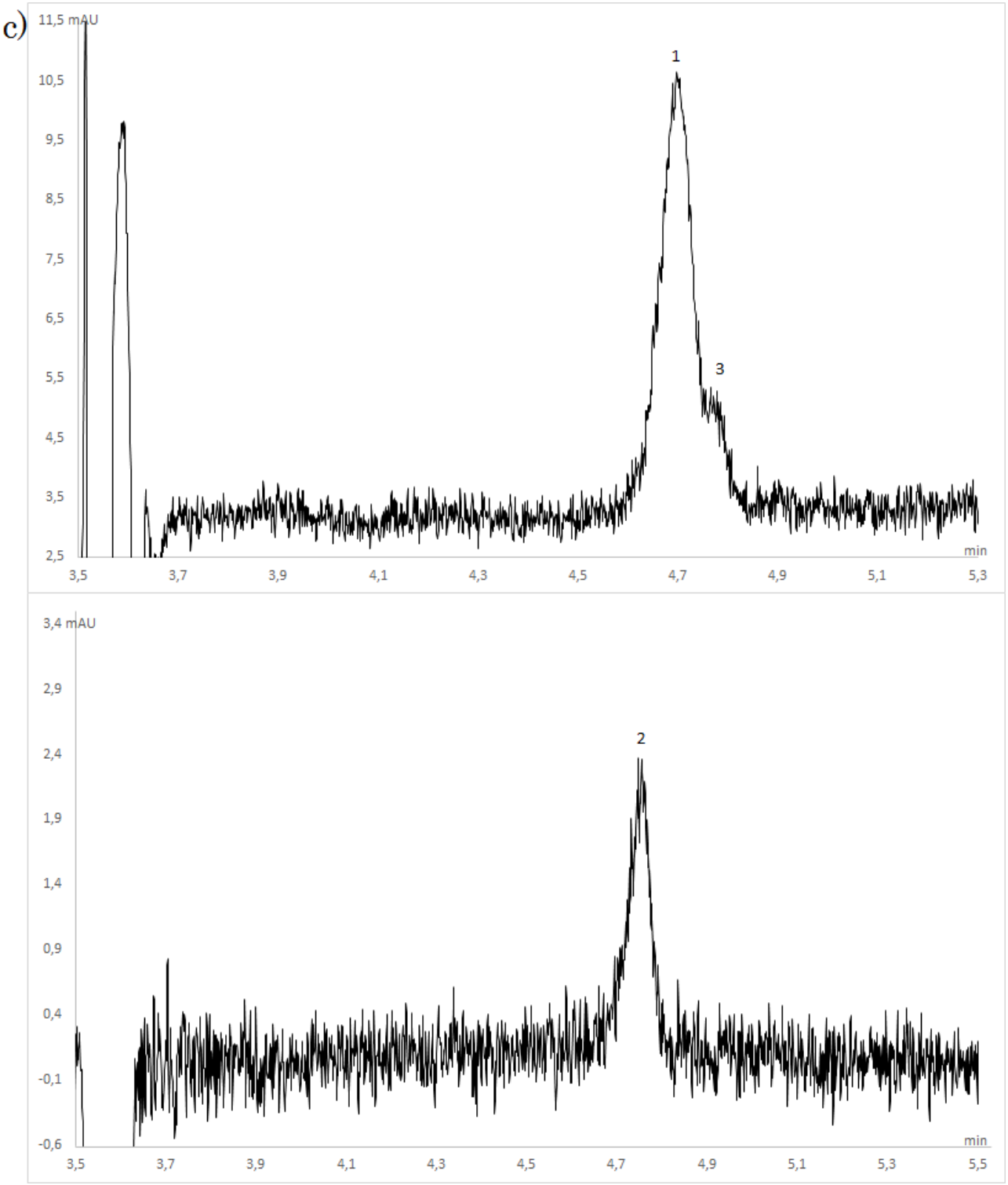
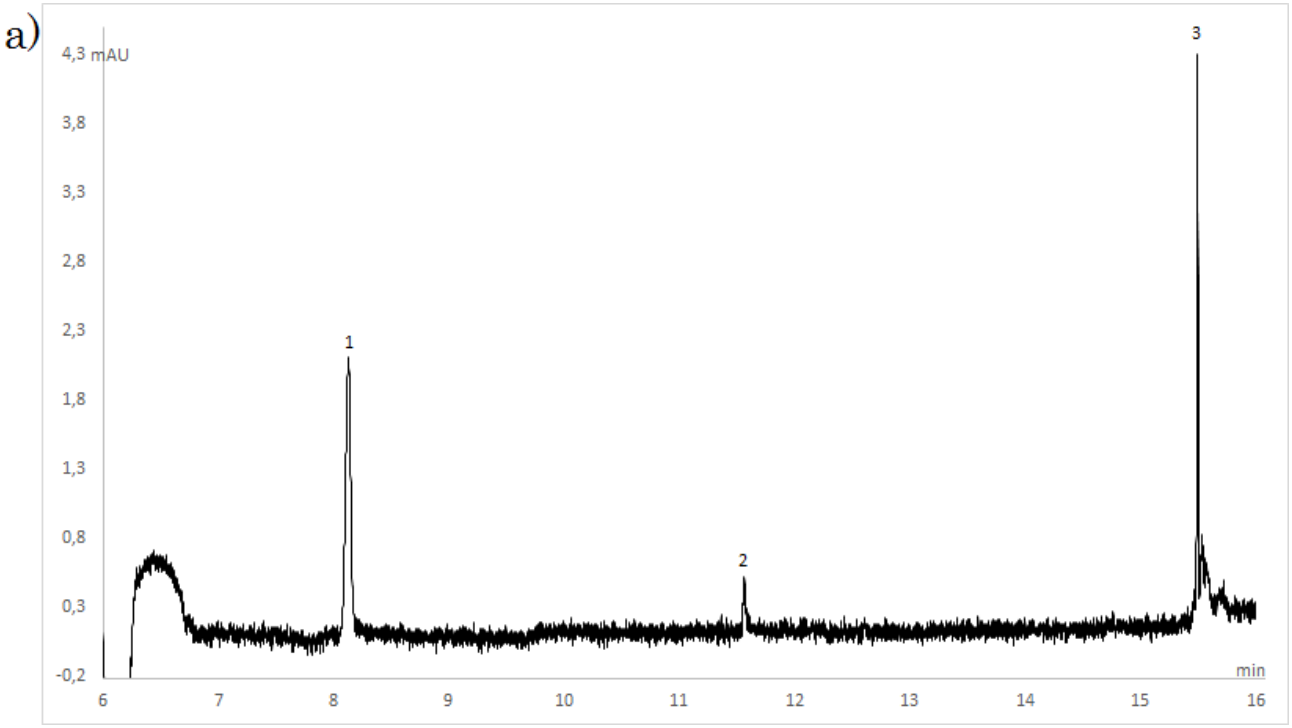
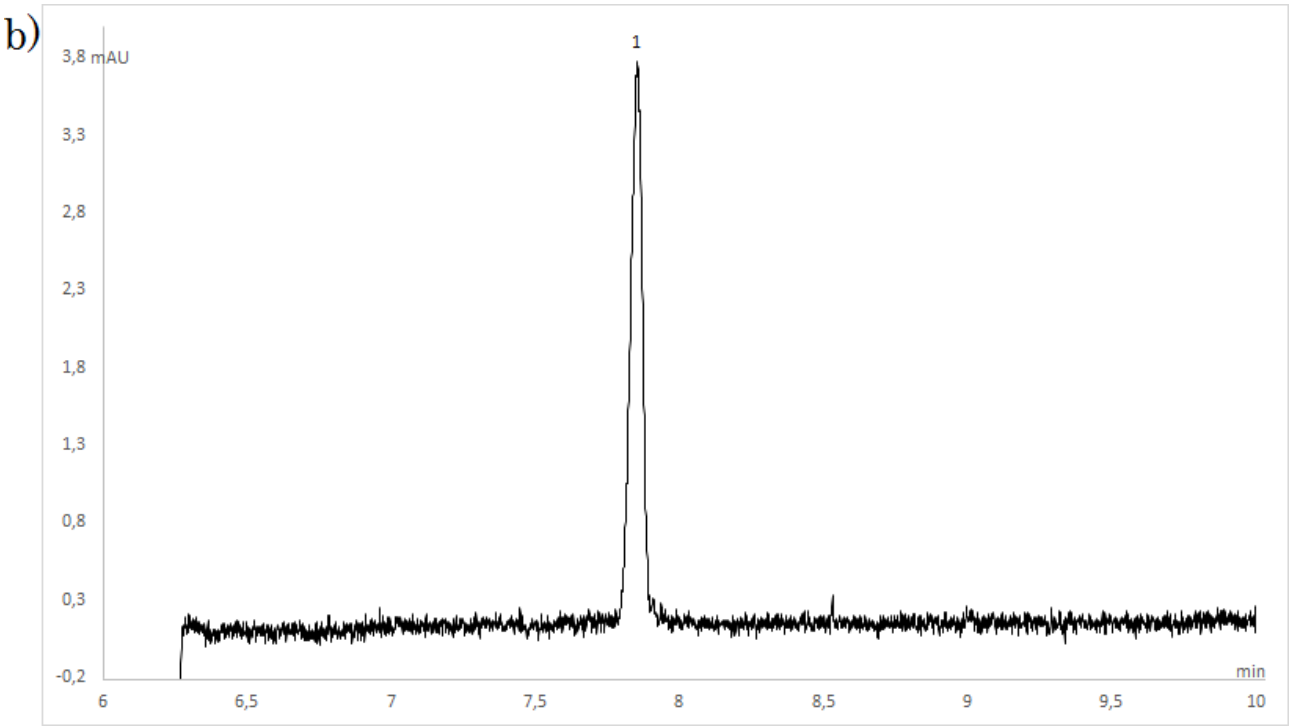


Figure 2. Capillary electrophoresis separation of steroid hormones. In PFMEKC, the migration order was T-gluc, fluoxymesterone, androstenedione, testosterone, 17α-hydroxyprogesterone, 17α-methyl testosterone, and progesterone. Standards at the 2 µg/mL concentration. Migration of electroosmosis is 5.2 min. Specific detection of the androgens and the progestogens at UV 247 (±2) nm. In CZE-CAPS, the migration order was E2-gluc (at 10 µg/mL), T-gluc (at 6.7 µg/mL), E1-gluc

(at 4 µg/mL), and E3-gluc (at 7.7 µg/mL). Detection wavelength was 200 (±5) nm for E1-gluc, E2-gluc, and E3-gluc. Detection of T-gluc was as in PF-MEKC. In CZE-AA, the migration order was E2-gluc (at 10 µg/mL), T-gluc (at 6.7 µg/mL), and E1-gluc (at 4 µg/mL). Detection wavelengths were as in CZE-CAPS. E3-gluc was not detected at 7.7 µg/mL concentration.





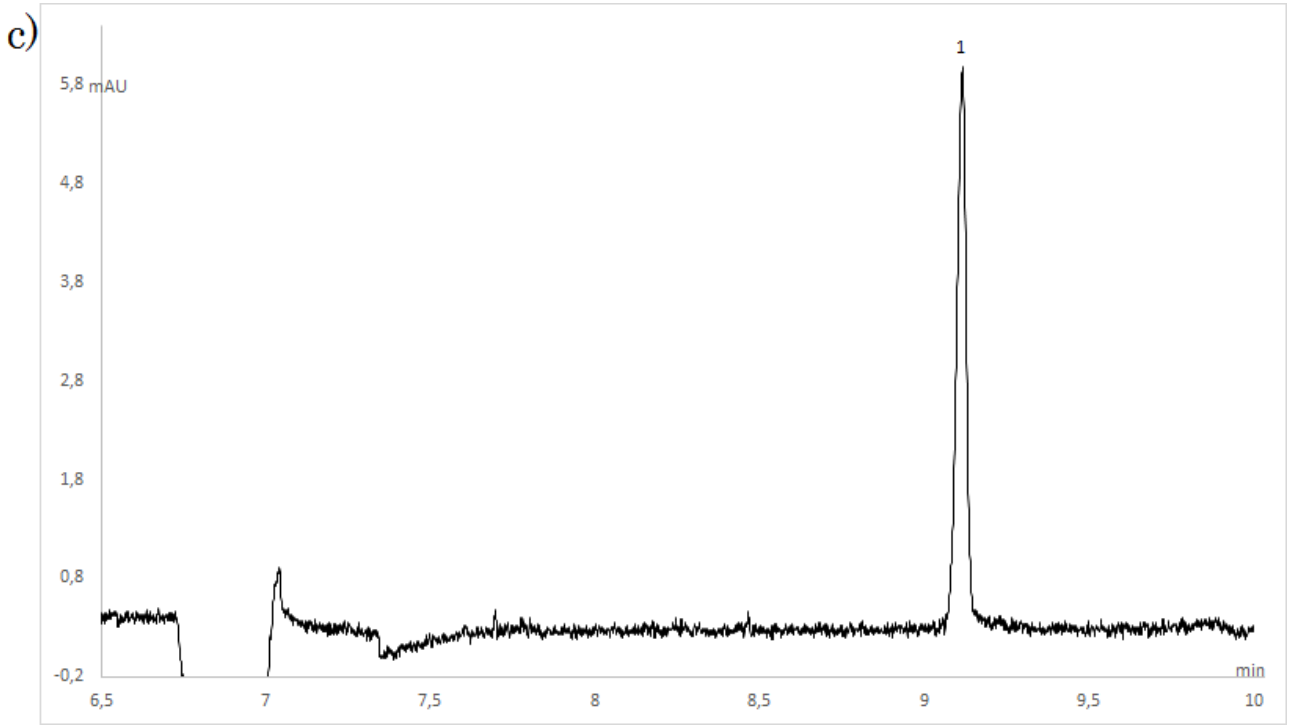


Figure 3. Electropherograms of analytical samples made from the precipitates of the influent water. Electropherograms are made with PFMEKC, CZE-AA, and CZE-CAPS. The deposit containing the soluble particles was produced on a membrane filter during filtration. The precipitate was macerated with ethyl acetate-methanol-water mixture. After concentration of the eluent, the steroids were separated. Compounds in the dissolved precipitate were T-gluc, Andr, and Prog. The details about the sample concentration and clean-up are in Fig. 1. Compounds were identified spiking with 2 μg/mL standards.

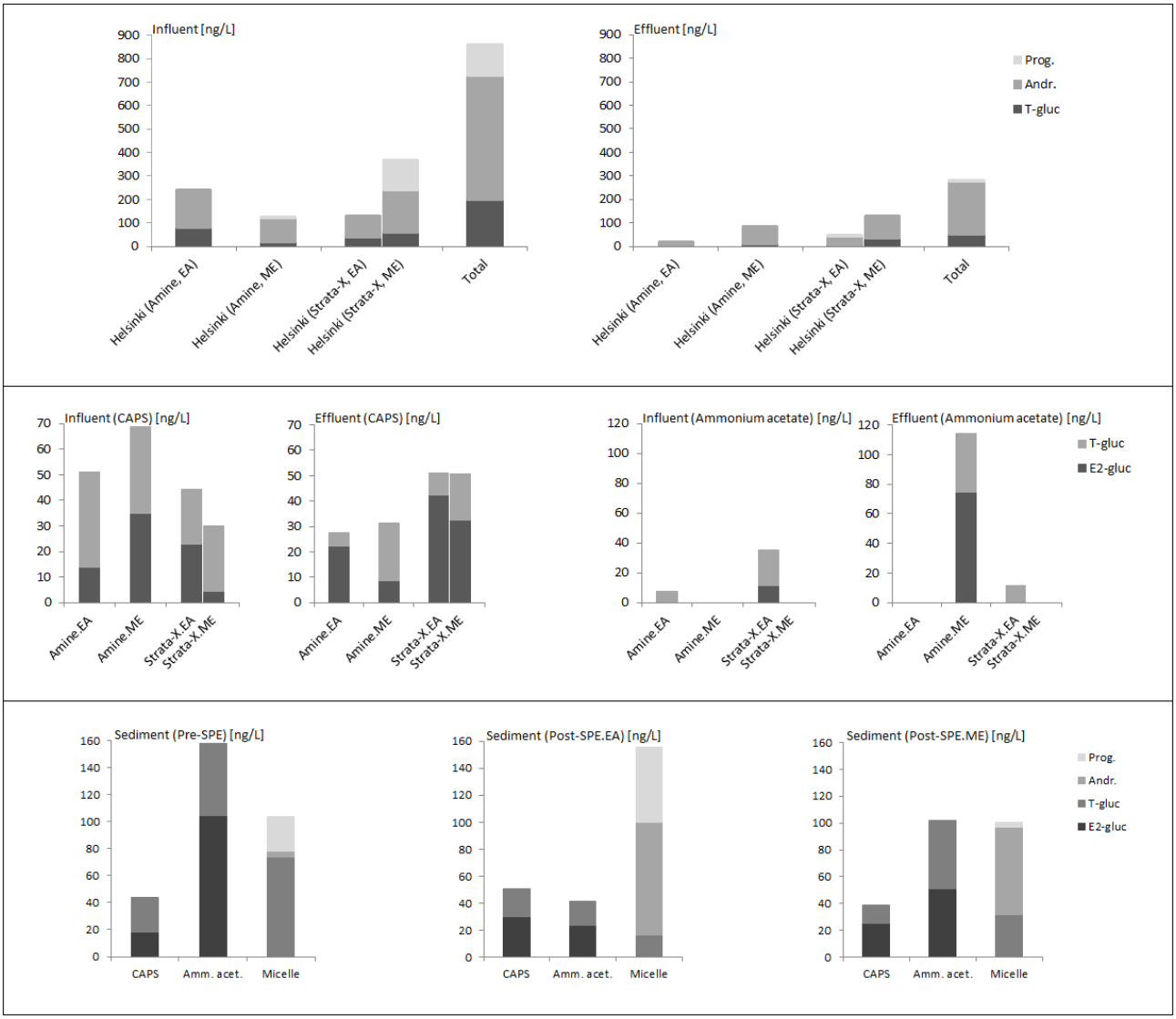


Figure 4 Concentrations of steroid hormones in the extracts of influent and effluent waters of Helsinki wastewater purification plant. Precipitates produced in filtration (pre-SPE, water filtrated when arrived in the laboratory; post-SPE (diluted filtrate which was pretreated with nonionic sorbent and eluted with ethyl acetate (EA) and methanol (ME), see Table 1). Sample preparation methods are as follows: Amino SPE, elution with EA; Amino SPE, elution with ME; Strata-X SPE, elution with ethyl acetate; Strata-X SPE, elution with methanol. Total amount in 1 L means the total concentration of all steroids analysed from the Strata-X eluents EA and ME after combining the steroid concentration in the amine eluent ethyl acetate or in the amine eluent methanol (total amount in 1 L:Strata-X(EA + ME) + Amine(EA) or total amount in 1 L:Strata-X(EA + ME) +Amine(ME)). The sample preparation procedure is described in the Extraction and preconcentration of analytes section. Calculations of the quantitative values are described in the B Preparation of standard solutions^ section and in Results section. The compounds are androstenedione (Andr), progesterone (Prog), testosterone-glucoside (T-gluc), and estradiol-glucoside (E2-gluc). The methods are CZE-CAPS, CZEAA (ammonium acetate), and PF-MEKC (micelle).

Table 1A. Electrophoretic mobility parameters of the steroid compounds used in PF-MEKC method. The measurements are done with a steroid mixture with five replicates. No sample concentration with SPE.

Name		EOF [min]	Migration time [min]	Electroosmotic flow [$m^2V^{-1}s^{-1}$]	Total velocity [$m^2V^{-1}s^{-1}$]
17α-hydroxyprogesterone	Mean	5.68	12.72	6.72-08	3.01E-08
	SD	0.08	0.59	9.14E-10	1.51E-09
	RSD (%)	1.4	4.7	1.4	5.0
17α-methyl testosterone	Mean	5.69	12.72	6.71E-08	3.01E-08
	SD	0.08	0.62	9.88E-10	1.55E-09
	RSD (%)	1.4	4.9	1.5	5.2
Androstenedione	Mean	5.77	10.88	6.62E-08	3.51E-08
	SD	0.21	0.60	2.49E-09	2.05E-09
	RSD (%)	3.6	5.5	3.8	5.8
Fluoxymesterone	Mean	5.76	10.74	6.62E-08	3.56E-08
	SD	0.20	0.58	2.43E-09	2.04E-09
	RSD (%)	3.5	5.4	3.7	5.7
Progesterone	Mean	5.68	12.81	6.72E-08	2.98E-08
	SD	0.08	0.64	9.12E-10	1.59E-09
	RSD (%)	1.4	5.0	1.4	5.3
Testosterone	Mean	5.76	12.79	6.63E-08	2.99E-08
	SD	0.21	0.88	2.51E-09	2.16E-09
	RSD (%)	3.6	6.9	3.8	7.2
T-gluc	Mean	5.77	7.81	6.62E-08	4.91E-08
	SD	0.21	0.59	2.46E-09	3.86E-09
	RSD (%)	3.6	7.5	3.7	7.9

Table 1B. Electrophoretic mobility of the steroid standards from the CZE results. The measurements are done using steroid mixtures. Measurements are done with five replicates. No sample concentration with SPE.

Name		EOF [min]	Migration time [min]	Electroosmotic flow [$m^2V^{-1}s^{-1}$]	Total velocity [$m^2V^{-1}s^{-1}$]
CZE-CAPS					
E2-gluc	Mean	8.64	11.63	4.43E-08	3.28E-08
	SD	0.44	0.26	2.24E-09	7.48E-10
	RSD (%)	5.1	2.3	5.1	2.3
T-gluc	Mean	8.64	12.16	4.43E-08	3.15E-08
	SD	0.44	0.78	2.24E-09	1.95E-09
	RSD (%)	5.1	6.4	5.1	6.2
CZE-AA					

E2-gluc	Mean	6.63	8.87	5.77E-08	4.34E-08	
	SD	0.39	0.90	3.37E-09	4.26E-09	
	RSD (%)	5.9	10	5.9	10	
T-gluc	Mean	6.63	9.02	5.77E-08	4.26E-08	
	SD	0.39	0.87	3.37E-09	3.96E-09	
	RSD (%)	5.9	9.6	5.9	9.3	

The mobility of electroosmosis is calculated from each of the analyses by using methanol as the neutral marker. Calculations made with the equation $\mu_{ep} = \mu_{tot} - \mu_{eo}$, $\mu_{tot} = (L_{det}L_{tot}) / (Ut_m)$ and $\mu_{eo} = (L_{det}L_{tot}) / (Ut_{eo})$, where μ_{ep} and μ_{eo} are the electrophoretic mobilities of the analyte and electroosmosis, L_{det} is the length of the capillary to the detector, L_{tot} is the length of the total capillary, U is the applied voltage during the analysis, and $_{tm}$ and t_{eo} are the migration times of the analyte and electroosmosis (from the electropherogram), respectively. CAPS (3-[cyclohexylamino]-1-propane-sulfonic acid; AA ammonium acetate.

Table 2A. Calibration data of the steroids with PF-MEKC.

Standard mixture calibration	**Steroid**	**Linear equation**	**R^2 value**	**Concentration range [µg/mL]**	**LOD [µg/mL]**	**LOQ [µg/mL]**
PF-MEKC	T-gluc	y = 1.089x + 0.001	0.996	0.5-8.0	0.50	1.50
Conc. 0.5-10.0 µg/mL	Fluoxymesterone	y = 0.468x + 0.022	0.966	0.5-8.0	0.50	1.50
	Androstenedione	y = 0.632x - 0.029	0.940	0.5-8.0	0.50	1.50
	Testosterone	y = 0.779x - 0.213	0.962	0.5-8.0	0.38	1.15
	17α-hydroxyprogesterone	y = 2.944x + 3.040	0.968	0.5-10.0	0.30	0.90
	17α-methyl testosterone	y = 1.150x - 0.354	0.947	0.5-6.0	0.07	0.21
	Progesterone	y = 4.315x - 4.077	0.968	0.5-10.0	0.11	0.32

LOD was measured from the electropherogram using peak height of known steroid concentration (S, signal) and average noise peak height (N, noise). LOQ was calculated from the corresponding experimental LOD-value multiplied by 3. S / N = 3 and LOQ = 3LOD.

Table 2B. Water samples. Calibration data of the steroids with CZE-CAPS and CZE-AA.

Standard addition method	**Steroid**	**Linear equation**	**R^2 value**	**Method concentration range [µg/mL]**	**Method LOD [µg/mL]**	**Method LOQ [µg/mL]**
CZE-CAPS Effluent Amine-SPE EA elution	E2-gluc	y = 3.422x + 1.5111	0.963	0.0-8.0	0.06	0.17
	T-gluc	y = 2.9086x + 0.3237	0.978	4.0-8.0	0.08	0.24
CZE-CAPS Effluent Amine-SPE ME elution	E2-gluc	y = 3.8396x - 0.6674	0.994	0.0-8.0	0.23	0.68
	T-gluc	y = 3.1651x - 1.4216	0.994	2.0-8.0	0.29	0.88

CZE-CAPS Effluent Strata -SPE EA elution	E2-gluc T-gluc	y = 2.6282x + 2.2298 y = 2.1219x + 2.4938	0.978 0.957	2.0-8.0 2.0-8.0	0.12 0.15	0.37 0.46
CZE-CAPS Effluent Strata SPE ME elution	E2-gluc T-gluc	y = 3.6309x - 2.3457 y = 2.8817x - 1.066	0.982 0.996	2.0-8.0 2.0-8.0	0.22 0.28	0.67 0.84
CZE-CAPS Influent Amine-SPE EA elution	E2-gluc T-gluc	y = 3.9724x + 1.1049 y = 2.9701x + 2.2041	0.938 0.950	2.0-8.0 2.0-8.0	0.06 0.08	0.19 0.25
CZE-CAPS Influent Amine-SPE ME elution	E2-gluc T-gluc	y = 4.912x - 3.4244 y = 4.0161x - 2.7281	0.976 0.977	2.0-8.0 2.0-8.0	0.25 0.31	0.75 0.94
CZE-CAPS Influent Strata SPE EA elution	E2-gluc T-gluc	y = 5.928x - 2.6892 y = 4.5953x - 1.9965	0.955 0.958	0.0-8.0 0.0-8.0	0.37 0.47	1.10 1.41
CZE-CAPS Influent Strata SPE ME elution	E2-gluc T-gluc	y = 4.1879x - 0.3555 y = 3.0716x + 1.5812	0.992 0.994	2.0-8.0 2.0-8.0	0.10 0.12	0.29 0.37
Standard mixture calibration	**Steroid**	**Linear equation**	**R^2 value**	**Concentration range [µg/mL]**	**LOD [µg/mL]**	**LOQ [µg/mL]**
CZE-AA Conc. 0.1-0.6 µg/mL	E2-gluc T-gluc	y = 8.4913x - 0.838 y = 9.6667x - 1.2108	0.917 0.l890	0.1-0.6 0.2-0.6	0.28 0.27	0.85 0.80

751 Calibration made with the method of standard addition. The calibration was made with five concentration levels.
752 CAPS: (3-[cyclohexylamino]-1-propane-sulfonic acid, AA: ammonium acetate, ME: methanol, EA: ethyl acetate.
753 LOD was measured from the electropherogram using peak height of known steroid concentration (S, signal) and
754 average noise peak height (N, noise). LOQ was calculated from the corresponding experimental LOD-value
755 multiplied by 3. S / N = 3 and LOQ = 3LOD.
756

757 Table 2C. Solid particle sample. Calibration data of the steroids with PF-MEKC, CZE-CAPS, and CZE-AA.

Standard addition method	**Steroid**	**Linear equation**	**R^2 value**	**Method concentration range [µg/mL]**	**Method LOD [µg/mL]**	**Method LOQ [µg/mL]**
PF-MEKC (pre SPE)	T-gluc Andr. Prog.	y = 2.0122x + 2.9608 y = 0.4377x + 0.0408 y = 1.0409x + 0.5366	0.957 0.988 0.997	2.0-8.0 2.0-8.0 2.0-8.0	0.24 0.87 0.17	0.73 2.61 0.50
CZE-AA (pre SPE)	E2-gluc T-gluc	y = 7.068x - 14.755 y = 4.7009x - 5.0426	0.942 0.985	2.0-8.0 2.0-8.0	0.31 0.48	0.94 1.45

CZE-CAPS (pre SPE)	E2-gluc	y = 3.9719x - 1.422	0.993	2.0-8.0	0.15	0.44
	T-gluc	y = 3.0304x + 1.5886	0.994	2.0-8.0	0.16	0.49
PF-MEKC (Strata-SPE, EA elution)	T-gluc	y = 4.6977x + 1.5708	0.952	2.0-8.0	0.19	0.56
	Andr.		0.932	2.0-8.0	1.67	5.00
	Prog.	y = 0.8812x - 1.4689	0.984	2.0-8.0	0.13	0.39
		y = 2.4304x - 2.6903				
PF-MEKC (Strata SPE, ME elution)	T-gluc	y = 4.8334x - 3.0582	0.974	2.0-8.0	0.38	1.13
	Andr.		0.979	2.0-8.0	2.00	6.00
	Prog.	y = 0.4893x - 0.6394	0.963	0.0-8.0	0.20	0.60
		y = 1.6483x - 0.1152				
CZE-AA (Strata SPE, EA elution)	E2-gluc	y = 6.4904x - 3.0443	0.955	2.0-8.0	0.17	0.50
	T-gluc	y = 4.8669x - 1.7457	0.960	2.0-8.0	0.23	0.69
CZE-AA (Strata SPE, ME elution)	E2-gluc	y = 10.996x - 11.261	0.972	2.0-8.0	0.14	0.43
	T-gluc	y = 8.2086x - 8.3505	0.951	2.0-8.0	0.21	0.62
CZE-CAPS (Strata SPE, EA elution)	E2-gluc	y = 5.3003x - 3.173	0.994	2.0-8.0	0.14	0.42
	T-gluc	y = 4.2069x - 1.7288	0.991	2.0-8.0	0.17	0.51
CZE-CAPS (Strata SPE, ME elution)	E2-gluc	y = 6.8599x - 3.4434	0.991	2.0-8.0	0.10	0.30
	T-gluc	y = 5.3748x - 1.4542	0.995	2.0-8.0	0.12	0.37

Calibration made with the method of standard addition. The calibration was made with five concentration levels. CAPS: (3-[cyclohexylamino]-1-propane-sulfonic acid, AA: ammonium acetate, ME: methanol, EA: ethyl acetate. LOD was measured from the electropherogram using peak height of known steroid concentration (S, signal) and average noise peak height (N, noise). LOQ was calculated from the corresponding experimental LOD-value multiplied by 3. S / N = 3 and LOQ = 3LOD.

Table 3. Effect of solvent used to elute steroid hormones from Strata-X and quaternary amine sorbents. Samples are influent and effluent waters from Helsinki plant. PF-MEKC and both CZE methods were used.

	Compounds			
Method	E2-gluc [ng/L]	T-gluc [ng/L]	Andr. [ng/L]	Prog. [ng/L]
PF-MEKC		*Influent*		
Amine / EA	na	80.6	157.7	nd
Amine / ME	na	17.4	104.0	2.1
Strata / EA	na	39.6	90.0	nd
Strata / ME	na	60.1	180.8	126.3
Total amount [ng/L]	na	197.7	532.5	128.4
		Effluent		

Amine / EA	na	1.0	17.6	nd
Amine / ME	na	10.1	73.3	nd
Strata / EA	na	8.3	35.9	4.8
Strata / ME	na	33.4	97.7	nd
Total amount [ng/L]	na	52.8	224.4	4.8
CZE-CAPS		*Influent*		
Amine / EA	13.9	37.1	na	na
Amine / ME	34.9	34.0	na	na
Strata / EA	22.7	21.7	na	na
Strata / ME	4.3	25.8	na	na
Total amount [ng/L]	75.5	118.5	na	na
		Effluent		
Amine / EA	22.1	5.6	na	na
Amine / ME	8.7	22.5	na	na
Strata / EA	42.4	8.8	na	na
Strata / ME	32.3	18.5	na	na
Total amount [ng/L]	105.5	55.3	na	na
CZE-AA		*Influent*		
Amine / EA	nd	7.5	na	na
Amine / ME	nd	nd	na	na
Strata / EA	11.3	23.7	na	na
Strata / ME	nd	nd	na	na
Total amount [ng/L]	11.3	31.1	na	na
		Effluent		
Amine / EA	nd	nd	na	na
Amine / ME	74.8	39.2	na	na
Strata / EA	nd	11.5	na	na
Strata / ME	nd	nd	na	na
Total amount [ng/L]	74.8	50.7	na	na

nd: not detected, na: not analyzed, CAPS: (3-[cyclohexylamino]-1-propane-sulfonic acid, AA: ammonium acetate.

Table 4. Results of the filtrates (precipitates from the filtration). Non-ionic steroids were determined with PF-MEKC and their conjugates determined with CZE methods. EA: ethyl acetate, ME: methanol.

	Compound			
	E2-gluc	T-gluc	Andr.	Prog.
Influent sediment: Pre-SPE				
CZE-CAPS				
x-axis intersection	0.358	0.524	-	-
Initial sample, c [ng/L]	17.9	26.2	-	-
c [ng/g]	4.5	6.7	-	-
CZE-AA				
x-axis intersection	2.087	1.073	-	-
Initial sample, c [ng/L]	104.3	53.6	-	-

c [ng/g]	26.5	13.6	-	-
PF-MEKC				
x-axis intersection	-	1.471	0.092	0.515
Initial sample,				
c [ng/L]	-	73.6	4.6	25.8
c [ng/g]	-	18.7	1.2	6.5
Influent sediment: Post-SPE, elution with EA				
CZE-CAPS				
x-axis intersection	0.599	0.411	-	-
Initial sample,				
c [ng/L]	29.9	20.5	-	-
c [ng/g]	7.6	5.2	-	-
CZE-AA				
x-axis intersection	0.469	0.357	-	-
Initial sample,				
c [ng/L]	23.5	17.9	-	-
c [ng/g]	6.0	4.5	-	-
PF-MEKC				
x-axis intersection	-	0.334	1.666	1.107
Initial sample,				
c [ng/L]	-	16.7	83.3	53.3
c [ng/g]	-	4.3	21.2	14.1
Influent sediment: Post-SPE, elution with ME				
CZE-CAPS				
x-axis intersection	0.502	0.271	-	-
Initial sample,				
c [ng/L]	25.1	13.5	-	-
c [ng/g]	6.4	3.4	-	-
CZE-AA				
x-axis intersection	1.025	1.017	-	-
Initial sample,				
c [ng/L]	51.2	50.9	-	-
c [ng/g]	13.0	12.9	-	-
PF-MEKC				
x-axis intersection	-	0.633	1.305	0.070
Initial sample,				
c [ng/L]	-	31.6	65.2	3.5

c [ng/g]	-	8.0	16.6	0.9

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